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Oral Toxicity of β-*N*-Acetyl Hexosaminidase to Insects

PATRICK F. DOWD,* ERIC T. JOHNSON, AND T. SCOTT PINKERTON

Crop BioProtection Research Unit, Agricultural Research Service, U.S. Department of Agriculture, National Center for Agricultural Utilization Research, 1815 North University Street, Peoria, Illinois 61604

Insect chitin is a potential target for resistance plant proteins, but plant-derived chitin-degrading enzymes active against insects are virtually unknown. Commercial β-*N*-acetylhexosaminidase (NAHA), a chitin-degrading enzyme from jack bean *Canavalia ensiformis*, caused significant mortality of fall armyworm *Spodoptera frugiperda* larvae at 75 μg/gm, but no significant mortality was noted with *Aspergillus niger* NAHA. Maize *Zea mays* callus transformed to express an *Arabidopsis thaliana* clone that putatively codes for NAHA caused significantly higher mortality of cigarette beetle *Lasioderma serricorne* larvae and significantly reduced growth rates (as reflected by survivor weights) of *S. frugiperda* as compared to callus that expressed control cDNAs. Tassels from model line Hi-II maize (*Z. mays*) plants transformed with the NAHA gene fed to *S. frugiperda* caused significantly higher mortality than tassels transformed to express glucuronidase; mortality was significantly correlated with NAHA expression levels detected histochemically. Leaf disks from inbred Oh43 maize plants transformed with the NAHA gene on average had significantly less feeding by caterpillars than null transformants. Leaf disks of Oh43 transformants caused significant mortality of both *S. frugiperda* and corn earworm *Helicoverpa zea* larvae, which was associated with higher expression levels of NAHA detected by isoelectric focusing, histochemically, or with antibody. Overall, these results suggest that plant NAHA has a role in insect resistance. Introduction of NAHA genes or enhancement of activity through breeding or genetic engineering has the potential to significantly reduce insect damage and thereby indirectly reduce mycotoxins that are harmful to animals and people.

KEYWORDS: Insect resistance; *Spodoptera*; *Lasioderma*

INTRODUCTION

Insects cause extensive economic losses to crops such as maize worldwide (*1*). Insect presence and damage also frequently lead to significantly increased levels of ear mold toxins (mycotoxins) in maize (*2*, *3*). Several studies have shown that in hybrids where *Bacillus thuringiensis* (Bt) crystal protein is expressed throughout the maize plant, the mycotoxin fumonisin occurs at much lower levels as compared to corresponding nonBt hybrids when European corn borers (*Ostrinia nubilalis* Hübner) occur at high levels and are the predominant ear pest (*4*−*8*). However, there are many different species of insects, predominantly beetles and caterpillars, that can damage maize ears and promote colonization by mycotoxin-producing fungi (*2*, *3*). Bt proteins are relatively selective, and high levels of production are needed to reliably kill heterozygous resistance gene-carrying insects (*9*, *10*) and indirectly reduce mycotoxin levels (*7*). On the basis of these criteria, several different Bt genes would be needed in order to satisfactorily control the multiple species of insect ear pests that can promote mycotoxins, levels of control that are not commercially available in maize hybrids at this time (*11*). There are also concerns that, despite resistance management through refugia for susceptible gene carrying insects, resistance to the Bt protein could eventually develop in insects feeding on transgenic plants expressing the protein as it has in insect species that were treated with spray formulations (*9*, *10*), which can also include cross-resistance to different Bt protein types (*12*). Thus, there is still a need for broad spectrum, multigenic resistance to insect ear pests of maize.

One strategy that has been used to produce multigenic pest resistance in plants is to introduce genes of different modes of action, a strategy that has met with mixed success (e.g., *13*, *14*). A more recent approach that has been suggested is to introduce a few genes that have different functional activities, which may promote additive or synergized resistance to insects equivalent to that for individually toxic, more species-specific genes such as Bt (*14*, *15*). For example, microbial chitinase degradation of the chitin membrane lining the insect gut appears to synergize the toxicity of Bt proteins (*16*). The effectiveness of microbial or plant-derived chitin-degrading enzymes, such as chitinase, that have been examined is limited (*16*), but recent studies have identified the first plant-derived chitinase gene that is effective against insects (*17*). An additional advantage of using chitin-degrading genes to control insects is that fungal pathogens may also be controlled (*14*). However, there is evidence that

* To whom correspondence should be addressed. Tel: 309-681-6242. Fax: 309-681-6686. E-mail: patrick.dowd@ars.usda.gov.

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certain levels of microbial chitinase expression can reduce the vigor (*18*) or alter growth patterns (*19*) of plants in which they are expressed.

Another group of chitin-degrading enzymes is the hexose aminidases, such as β-*N*-acetyl hexosaminidases (EC 3.2.1.52) (NAHAs), which hydrolyze terminal nonreducing *N*-acetyl-D-hexosamine residues from *N*-acetyl-D-hexosaminides (*20*). The substrate range of characterized hexosaminidases appears to be different than that of chitinases (IUBMB EC 3.2.1.14), which randomly hydrolyze *N*-acetyl-D-glucosamine (*20*). In addition, recent reports have indicated that NAHAs alone may be involved in fungal resistance (*13*). Our preliminary studies (*21*) suggested that NAHAs may be active against insects as well. We now report on the relative activity of some commercially available NAHAs against representative insects and the activity against insects of a NAHA coded for by an *Arabidopsis*-derived gene when expressed in plant systems, including regenerated maize plants.

MATERIALS AND METHODS

Insects. Laboratory colonies of fall armyworms (*Spodoptera frugiperda*) were reared on pinto bean-based diet at 27 ± 1 °C, 50 ± 10% relative humidity, and a 14:10 light:dark photoperiod (*22*). First instar larvae of *S. frugiperda* were used in bioassays. Laboratory colonies of cigarette beetle larvae (*Lasioderma serricorne*) were reared on a corn meal flour mix under the same conditions as the caterpillars as described previously (*23*), and second instar larvae were used in bioassays.

Plants. A culture of black Mexican sweet (BMS) maize cells originally obtained from P. Chourey (U.S. Department of Agriculture, Florida) was maintained on media at 28 ± 1 °C in the dark with 130 rpm shaking as described previously (*24*). The first batch of regenerated Hi-II plantlets received from the Iowa State University Plant Transformation Facility was transplanted into 8 L pots of RediEarth mix containing 250 mL by volume of Osmocote (both from Scotts Grace Sierra, Milpitas, CA) and was watered weekly with 250 mL of a 0.1 M $CaSO_4$−$MgSO_4$ solution. For the second batch of Hi-II transformants, the same soil mix was used, except the Osmocote 14:14:14 (Scotts Sierra Hort Products Co., Marysville, OH) was placed only in the bottom half of the pot (to help reduce problems with pathogens), 125 mL of a 44 mL/L stock Sprint 330 iron chelate micronutrient (Becker Underwood, Ames, IA) was added to each pot, and an initial treatment of liquid limestone (Limestone F, Cleary Chemical Corp., Dayton, NJ) at a rate of 1 L of a 10 mL/L composition was used as a single treatment to replace the multiple treatments of the Ca−Mg solution. To further reduce potential root pathogens, for the Oh43 transformants, the soil composition was modified to a mixture consisting of a lower half of SB330 bark mix (Sungro Horticulture, Bellevue, WA) Osmocote, Sprint 330, and liquid limestone, with the top layer of Redi Earth. Plants were watered with deionized water.

Transformed plantlets were removed from Petri dishes when leaves were 2 cm or longer. Plantlets were initially covered with a 300 or 600 mL clear plastic cup with 3 cm square holes cut on either side and covered with organdy held in place with hot glue. Cups were used to acclimate the plants and removed after the leaves reached the top of the cups. Hi-II model variety maize plants were held in the greenhouse with 18 ± 2 °C day and 24 ± 2 °C night temperatures (the greenhouse room had supplemental air conditioning) and at 50 ± 10% relative humidity. Oh43 inbred plants were held under the same conditions in a walk-in plant growth facility with alternating 1000 W sodium and halide lighting.

Bioassays with Commercial Materials. NAHA from jack bean (*Canavalia ensiformis*) and the fungus *Aspergillus niger* were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Prebuffered solutions containing the NAHAs were diluted in water to yield concentrations of 75 μg/gm. The same solutions without the enzymes were used as controls. Pinto bean-based insect diet disks were prepared by "casting" slabs of diet 1 mm thick, punching out 8 mm diameter disks, and freeze drying. Freeze-dried diet disks were stored in a sealed container in the refrigerator until bioassay setup. Each disk weighed approximately 15 mg after it was freeze-dried. Petri plates with tight-fitting lids (Falcon 1006, Becton Dickinson, Franklin Lakes, NJ) containing 5 mL of 3% water agar were used as bioassay containers. A 1 cm diameter Teflon disk (Scientific Specialties Service, Hanover, MD) was added to each plate, and the diet disks were placed on top of the Teflon disks. Twenty five microliters of NAHA solutions or control buffer solutions was added to each disk. The Petri dishes were placed in the refrigerator overnight to allow the solution to equilibrate throughout the disk and then used in bioassays. Ten newly hatched caterpillar larvae were added to each dish, and each treatment was setup in duplicate. Each disk provided sufficient material for ad libitum feeding by caterpillars over the duration of the assay period. The dishes were incubated under the same conditions used in insect rearing except plates were kept in the dark. Dishes were examined for larval mortality, and after 3 days, dishes were frozen until survivors of the bioassay could be weighed. Larvae were weighed to the nearest 0.01 mg using a Mettler AE163 analytical balance (Mettler Instrument Corp., Highstown, NJ). Assays with regenerated transformed callus tissue were run in a similar manner, except a callus clump ca. 1 cm in diameter and 3−5 weeks after transfer was placed on the Teflon disk. Ten caterpillars or cigarette beetle larvae were placed in each dish, and there was sufficient material for ad libitum feeding by the insects over the duration of the assay period.

Bioassays with Plant Material. Regenerated Hi-II plant tissue bioassays were performed with tassel material due to the small size of the plants, a high caterpillar mortality on leaves for all plant types, and a limited amount of caterpillar feeding on larger leaves initially noted for all plant types. The target tassel stage used in bioassays had spread tassel branches but no anther release. In some cases, tassels slightly earlier or later were used in order to have comparative control material [regenerated glucuronidase (GUS) transformants] available. Approximately 3 cm sections of tassel from the tip were removed and placed individually in the Petri dishes with 10 newly hatched *S. frugiperda* larvae. Material supplied was sufficient for ad libitum feeding by larvae over the duration of the assay period. Mortality was determined after 2 days. While setting up bioassays, the basal portion of the main tassel stem (below any branches or anthers) was removed to be used for histochemical analyses, and the remaining tassel was frozen in liquid nitrogen and stored at −80 °C for subsequent analyses (such as Southern blots). In cases where tassel material was limited, leaf or husk tissue was stored to be used as a DNA source.

Regenerated Oh43 bioassays used leaf tissue as plants were larger as compared to Hi-II and caterpillars would readily feed on larger leaves. A 4.25 cm diameter #1 qualitative filter paper disk (Whatman International, Maidstone, England) was placed in each Petri dish, and 120 μL of sterile distilled water was added to each to prevent drying out of leaf disks. Typically, the seventh leaf from the base was used in bioassays after the 10th leaf was visible (third leaf from the tip). A few B73 leaves were also used in bioassays for comparison. Leaf disks 2 cm in diameter were cut from each leaf as near the leaf tip as possible, plus 8 mm leaf disks were cut from the remaining leaf tip for later protein or RNA analysis. Leaf disks were placed individually in each dish along with 10 newly hatched caterpillars. Material provided was sufficient for ad libitum feeding by the caterpillars over the duration of the assay period. Bioassays were rated for feeding damage by determining total 0.25 mm^2 and 1 mm^2 feeding holes or equivalent areas by eye as described previously (*23*, *25*). Mortality and survivor weights were determined as just described. Oh43 tassels that had finished shedding pollen were frozen and then extracted for DNA or RNA as needed (see below).

Preparation of cDNA Construct for Transformation. The cDNA construct was prepared for biolistic transformation by excising the GUS region of pAHC25 and ligating in the NAHA gene, as described previously for other genes (*24*). The original plasmid containing the NAHA gene from *Arabidopsis thaliana* (Genbank BT000920) was obtained from the *Arabidopsis* DNA Biological Resource Center as stock #C105247. This gene contained the three regions (*26*) commonly conserved in NAHAs. A 5′ SmaI cut site and a 3′ SstI cut site were added immediately prior to and after the initiating ATG and terminating

TGA codons using the primers 5′-gccgcccgggatggttgagtatgatatca-3′ and 5′-gccggagctctcactgagcatagcaaga-3′, respectively. Pfu was used according to the manufacturer's instructions (Stratagene USA, La Jolla, CA), and thermal cycling conditions were the same as described previously (*24*) except that the annealing temperature was 60.5 °C. The product was purified using 0.8% agarose, and then, DNA was extracted from the gel using a binding matrix according to the manufacturer's instructions (Q-Biogene, Carlsbad, CA). The purified polymerase chain reaction (PCR) product was sequentially digested with SmaI and SstI and repurified as just described. The purified digestion product was ligated into similarly digested and purified pAHC25, after the GUS construct was similarly removed, using Rapid Ligase according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN).

The ligation product was introduced into DH5α strain of *Escherichia coli*, and putative transformants were selected on media containing 0.5% ampicillin as described previously (*24*). Colonies were restreaked onto LB−ampicillin media, and then, individual colonies were inoculated into Terrific broth containing 0.5% ampicillin and incubated with shaking at 37 °C for 18 h. Plasmid DNA was extracted and purified using Qiagen kits according to the manufacturer's instructions (Qiagen, Inc., Valencia, CA). Purified plasmid DNA was digested with SmaI and SstI as just described, and the products were separated on 0.8% agarose by electrophoresis. Plasmid DNA that yielded appropriately sized products was sequenced to verify the sequence of the NAHA using ABI kits and an ABI 3100 sequencer as described previously (*24*). Plasmids with the correct sequence were used for transformation. Another putative NAHA gene was cloned from *Arabidopsis* Columbia O strain based on sequence Genbank sequence NM 100439 using 150 ng of plant-derived DNA (see below), the primers 5′-gccgcccgggatgctaactcttttcaagttt-3′ and 5′-gccggagctcttattgatcttgagcacc-3′, and thermal cycling conditions as described above (*24*). This gene also had the three conserved regions of NAHAs. Constructs were developed and verified as just described. In this case, the PCR product was about 70 base pairs larger that the expected product as later determined by sequencing of the completed construct, suggesting that an intron was present.

Transformations and Regenerations. Maize transformation and regeneration into plantlets were performed by the Iowa State Plant Transformation Facility. BMS cell transformation and selection using the stock center clone and genomic DNA clone were performed as described previously (*24*). Briefly, 4 μL of plasmid DNA was coated on to gold particles and introduced into BMS cells with a PDS-1000 Biolistic Device (Bio-Rad Laboratories, Hercules, CA) with 1100 psi rupture disks. Cells were allowed to recover on BMS media overnight, placed on selection media containing 3 g/L gelrite and 1 mg/L bialaphos, and transferred every 3−4 weeks. Once clumps of callus ca. 1 cm in diameter became available, they were used at various intervals for DNA, enzyme, and bioassay determinations (typically 4−6 weeks after transfer).

Verification of NAHA DNA Presence and mRNA Production. The presence of the introduced NAHA gene was verified in callus, Hi-II, and Oh43 transformants. Genomic DNA was extracted from tassel material of Hi-II transformants using the DNA easy kit (Qiagen, Inc.) and from callus and Oh43 leaves using the Wizard Genomic DNA purification kit (Promega, Madison, WI) according to each respective manufacturer's instructions. PCR analysis was run using Roche PCR master mix kit (Roche Applied Science) according to the manufacturer's instructions and 150 ng of genomic DNA, 50 ng of each primer, and 5% dimethyl sulfoxide in final 50 μL reaction mixes. Primer pairs used in verifying the presence of the gene were the same as those originally used for preparation of the insert for ligation.

The presence of the correct sequence could be tentatively verified based on the size of the product. Further confirmation was undertaken by digesting gel-purified PCR products with BamHI and examining the size of the digestion productions. (Digestions were only done with Hi-II material.) A representative number of PCR products from both Hi-II and Oh43 genomic DNA were also checked for the correct sequence by trapping the product using a TOPO TA cloning kit and transforming Top10 cells according to the manufacturer's instructions (InVitrogen Corp., Carlsbad, CA). Plasmid DNA isolated from positive transformants was sequenced using M13 forward and reverse primers supplied with the kit and evaluated as described above. Representative larger scale genomic DNA preps from both Hi-II and Oh43 transformants were subjected to Southern blot analysis using 15−20 μg genomic DNA per well according to Southern (*27*). The NAHA insert was labeled and used as the probe, and the genomic DNA was digested with SmaI, SstI, or EcoRI. DNA was detected using the Genius System according to manufacturer's instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN).

The presence of NAHA mRNA in Oh43 was verified using reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted using the SV Total RNA Isolation kit according to the manufacturer's instructions (Promega Corp.). Typically, two leaf disks taken from the same leaf used in bioassays gave the most consistent yield of RNA. The cDNA was generated from the RNA using ProStar Ultrahigh Fidelity RT-PCR mix (Stratagene USA) according to the manufacturer's instructions. The resulting cDNA was used as a source of DNA to indicate the translation of the NAHA gene as described above for gene confirmation from genomic DNA. Maz95 primers 5′-cacgggtgtgatggttggaa-3′ and 5′-atgccagggaacatggtcgt-3′ were used as a positive control and also to confirm that only cDNA generated from mRNA was present because the primers were so designed that they ran through a portion of the gene that, if genomic DNA were present, a larger product (923 bp vs 800 bp) would be generated due to the presence of an intron in the genomic DNA. PCR conditions were the same as those described previously (*24*) except that a 60.5 °C annealing temperature and 40 cycles were used.

Assay for NAHA Presence. Tassel stem sections of Hi-II were analyzed for the presence of GUS and NAHA using histochemical methods. The GUS activity was detected according to previously published procedures (*28*) at 35 °C in a 150 μL total volume per well in 96 well plates. NAHA was detected using a coupling assay including 25 μL of a 10 mg/mL solution of naphthol AS-BI *N*-acetyl β-D-glucosamide (NAGA) in dimethylformamide and 25 μL of a 50 mg/mL solution of fast blue BB salt dissolved in water (Sigma-Aldrich Chemical Co.) to 100 μL of pH 7.4, 0.1 M sodium phosphate buffer containing a ca. 2 mm section of the tassel stem. Assays were run using 96 well plates that were shaken at 20 rpm at 35 °C. Plates were examined at 1, 2, and 4 h and overnight and rated for relative intensity of response on a 1−4 scale with 1 = no detectible response, 2 = some response, 3 = moderate response, and 4 = strong response. Applicability of the assay was verified using jack bean seed pieces grown from seed obtained from The Banana Tree (Easton, PA), which produced a purple color. A section of each stem was also incubated with fast blue alone to act as a relative background control. High background seen with Oh43 tassel material (presumably due to high phenolic content) limited the usefulness of this type of an assay as a relative indicator of NAHA activity, so other methods were used with Oh43 material (see below).

Isoelectric focusing of enzyme preparations from tissues was performed as described previously using pH 3.5−9.5 precast gels (GE Healthcare Bio-Sciences, Piscataway, NJ) (*24*, *29*). Gels were stained for NAHA activity using the same conditions as described for histochemical assays, although quantities of materials were proportionally increased to accommodate the 100 mL volume of the staining dish. Commercial jack bean NAHA was used as a positive control and to determine relative activity through serial dilutions and yielded purple bands.

Detection of NAHA with Antibody. The last 20 amino acids coded for by the stock center *Arabidopsis* NAHA sequence (N-term-PVDNFYARRPPLGPGSCYAQ-C-term) had a high antigenicity index when examined by the Protean module of Lasergene DNA and protein analysis software (DNAstar, Madison, WI) and so were submitted for antibody preparation in rabbits to Bethel Laboratories (Montgomery, TX). Leaf disks from Oh43 leaves were homogenized and clarified as described for enzyme detection except that PVPP and dithiolthreitol were also added to the buffer. Apparatus and materials for all subsequent work were from InVitrogen Corp. and were performed according to the manufacturer's instructions. Supernatants were diluted 3:1 in NuPage LDS sample buffer containing 20% β-mercaptoethanol and heated at 90 °C for 5 min. Sample preparations containing 3−5 μg of protein (depending on the batch) were applied to wells in 1 mm × 12

Table 1. Activity of Commercial NAHA Preparations against *S. frugiperda*[a]

	% mortality		*P* value			
treatment	D2	D3	D2	D3	weight (mg)	*P* value
			trial 1			
buffer control	0.0	0.0			2.93 ± 0.42	
jack bean NAHA	47.1	70.0	<0.0001	<0.0001	0.73 ± 0.25	0.009
			trial 2			
untreated	0.0	0.0			ND	
buffer control	0.0	0.0			ND	
jack bean NAHA	52.9	92.3	<0.0001	<0.0001	ND	
			trial 3			
buffer control	0.0	0.0			1.06 ± 0.12	
A. niger NAHA	0.0	0.0	NS	NS	0.77 ± 0.13	NS

[a] Weights are means ± standard errors. *P* values for mortality are based on χ^2 analysis, respectively, day 2 and day 3 values are 10.98 and 17.50 for trial 1 and 34.00 and 28.00 for trial 2. *P* values for weights are based on ANOVA, and respective *F* values are 9.81 for trial 1 and 2.17 for trial 3. ND, not determined; NS, not significant at $P < 0.05$. Ten larvae were set up with each replicate (see text).

lane NuPage 12% polyacryamide gel (PAG) Bis-Tris gels and separated using a Novex minicell with MOPS sodium dodecyl sulfate (SDS) running buffer at 200 V for 50 min. Gels were blotted onto PVDF Invitrolon membranes using Novex Xcell Blot Module and NuPage transfer buffer containing 10% methanol at 30 V for 1 h. NAHA was detected using the ECL Plus Western Blotting Detection System, with the antiNAHA serum diluted 1:2000 and horse radish peroxidase antirabbit secondary antibody (Bethel Laboratories) diluted 1:10000. The jack bean NAHA was also detected by the antiserum and was used as a standard to determine relative quantities of *Arabidopsis* NAHA present in the leaf disks. Relative quantities of NAHA were calculated by scanning film negatives of blots with an Imagemaster VDS system (Amersham Pharmacia Biotechnology, Piscataway, NJ) and using Imagemaster 1D software (Amersham Pharmacia Biotechnology) (*25*).

Statistical Analyses. Significant differences in mortality or other percentage data were determined by χ^2 analysis using PROC FREQ (*30*). In cases where the cell numbers were less than 5, the log likelihood ratio statistic was used within the same program (*30*). Significant differences in feeding ratings or weights were determined by analysis of variance (ANOVA) using PROC GLM or equivalent (*30*). Correlations between levels of NAHA and insect effects were determined using PROC REG (*30*).

RESULTS

Activity of Commercial NAHA and NAHA Expressing Callus against Insects. The jack bean NAHA was the more toxic of the commercial forms tested, causing overall ca. average 80% mortality of *S. frugiperda* after 3 days based on two different trials, which was significantly higher than mortality seen with buffer controls (**Table 1**). The NAHA from *A. niger* was less toxic and did not cause any significant mortality but did cause reductions in growth rates (based on survivor weights) of ca. 27%, which were not statistically significant at $P < 0.05$ as compared to buffer controls. The transformed callus did not cause any significant reductions in growth relative to the GUS control material, but *S. frugiperda* larvae that fed on the NAHA tissue were significantly smaller than those that fed on callus derived from an intron-containing NAHA gene introduction **(Table 2)**. A significantly higher number of *L. serricorne* larvae fed NAHA callus died as compared to those fed GUS callus by day 3 (**Table 2**).

Activity of Regenerated Hi-II Transformants Expressing NAHA against Insects. Mortality of insects fed Hi-II tassel tissue from putative NAHA and GUS transformants was variable. Some data had to be discarded as insecticide treatments for thrips in the second batch of plants had some residual effects for longer than expected (data not shown). Several NAHA transformant tassels caused high mortality to *S. frugiperda* as compared to GUS transformants, which was typically low (**Table 3**). Overall mortality of *S. frugiperda* larvae fed NAHA tassels in both series of Hi-II assays was significantly higher than that for larvae fed GUS tassels (**Table 3**). The NAHA activity could be readily detected in many transformants in tassel stems when stained histochemically (**Figure 1**). Mean feeding values were significantly lower for *S. frugiperda* larvae fed tassels in both sets of Hi-II assays as compared to those fed GUS tassels (**Table 4**).

Activity of Regenerated Oh43 Transformants Expressing NAHA against Insects. Leaf disks from some Oh43 NAHA transformants caused significant mortality of *S. frugiperda* larvae

Table 2. Effect of Callus with *Arabidopsis* NAHA Gene on Insects[a]

	% mortality		*P* value			
treatment	D1	D2	D1	D2	weight (mg)	*P* value
			S. frugiperda			
GUS	0.0	0.0	NS	NS	0.41 ± 0.05	NS
NAHA	0.0	0.0			0.38 ± 0.04	
intron NAHA	0.0	0.0	NS	NS	0.60 ± 0.05	0.0012
			L. serricorne[b]			
GUS	28.6	60.0	NS	0.013	ND	
NAHA	60.0	94.7		ND		
intron NAHA	25.0	76.5	NS	NS	ND	

[a] Weights are means ± standard errors. [b] Values for *L. serricorne* are days 2 and 3. *P* values for mortality are based on χ^2 analysis of NAHA vs GUS or NAHA vs intron NAHA; respectively, the day 3 value is 6.19 for *L. serricorne* GUS vs NAHA. *P* values for weights are based on ANOVA of NAHA vs GUS or NAHA vs intron NAHA; respectively, *F* values are 12.73 for NAHA vs intron NAHA for *S. frugiperda*. ND, not determined; NS, not significant at $P < 0.05$. Ten larvae were set up with each replicate (see text).

Table 3. Relative Activity of Representative Regenerated Plant Tissues with *Arabidopsis* NAHA on Fall Armyworm (*S. frugiperda*)

line no.	gene	% mortality	damage rating
		Hi-II batch 1	
15-2-1	yes	100	1[a]
29-1-2	yes	100	1
23-2-1	yes	50	3
GUS-1-2	no	11	10
GUS-2-2	no	0	9
		Hi-II batch 2	
52-2-3	yes	100	0[a]
44-1-2	yes	88	0
48-2-TP-3	yes	88	0
2C4-1-TP-1-3	no	0	7
2C5-1-TP-4	no	0	7
		Oh43	
2-1	yes	25	17[b]
4-2	yes	71	4
4-3	yes	33	12
7D1	yes	20	11
8-1-3	no	0	50
8-3-1	no	0	58

[a] The overall mortality for batch 1 of Hi-II NAHA vs GUS is 41.7 vs 16.1%, respectively, $P < 0.0001$, and χ^2 value = 22.33. The overall mortality for batch 2 of Hi-II NAHA vs GUS is 65.9 vs 39.5%, respectively, $P < 0.0001$, and χ^2 value = 57.36. [b] The overall mortality for Oh43 positive vs negative transformants is 6.4 vs 1.1%, respectively, $P = 0.03$, and χ^2 value of 4.56; likelihood ratio χ^2 values were used because one cell had less than five values. Ten larvae were set up with each replicate (see text).

Table 4. Mean Damage Rating Values of Caterpillars Caged with Positive and Negative Transformant Hi-II and Oh43 Maize Plant Material[a]

transformant type	feeding rating (N)	*P*	*F*
	Hi-II tassel set 1		
positive	5.3 ± 0.7 (29)		
negative (GUS)	8.3 ± 0.6 (15)	0.008	7.67
	Hi-II tassel set 2		
positive	1.9 ± 0.4 (72)		
negative (GUS)	5.8 ± 0.7 (28)	<0.0001	27.86
	Oh43 leaf		
positive	30.1 ± 1.6 (60)		
negative	48.0 ± 3.8 (9)	0.0002	15.91

[a] Values are means ± standard errors after 2 days. Feeding values for Hi-II are numbers of holes in tassels, and feeding values for Oh43 are number of mm^2 holes in leaf disks. Analyzed with ANOVA. Ten larvae were set up with each individual replicate (see text).

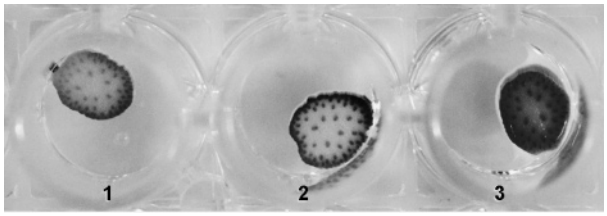

Figure 1. Histochemical detection of GUS and NAHA activity in tassel stems of Hi-II transformants. Key: 1, untransformed line that did not stain for GUS or NAHA in GUS staining solution; 2, GUS transformant that stained well for GUS; and 3, NAHA transformant that stained well for NAHA.

as compared to null transformed Oh43, which were associated with reductions in feeding rates relative to cDNA negative transformants (**Table 3**). Six transformants caused 20% or greater mortality, with two causing over 70% mortality. Additionally, more positive transformants had significantly smaller larvae as compared to negative transformants (data not shown). Overall mortality of *S. frugiperda* was significantly greater (6.4 vs 1.1%, $P = 0.03$, LLR χ^2 value = 4.57, respectively) for insects fed NAHA positive vs negative transformant leaf disks. Feeding on NAHA positive transformants was significantly less (30.1 ± 1.6, 48.0 ± 3.8, $P = 0.0002$, $F = 15.91$, respectively, day 2) (**Table 4**) than for negative transformants.

Detection of NAHA in Transformants. Both GUS and NAHA activity could be histochemically detected in tassel stem sections of respective Hi-II transformants (**Figure 1**) but was not seen in untransformed Hi-II material. Apparently due to higher phenolic content in tassel stems, histochemical assays could not readily distinguish NAHA activity in Oh43 tassel stems due to high background staining. NAHA activity was readily detected in the embryos of some seed of transformants that caused *S. frugiperda* mortality but was very faint in untransformed Oh43 (**Figure 2**). NAHA activity was faintly noted in the callus and some leaf disk homogenates from material active against insects that were subjected to isoelectric focusing, with a pI of approximately 4.8 (data not shown). NAHA could be detected with antibody in many Oh43 transformants (**Figure 3**). Up to ca. 150 μg/gm (20 ng/μg soluble protein) was detected in some lines tissue (based on jack bean NAHA equivalents), and 50 μg/gm was not uncommon.

Correlation of NAHA Levels with Insect Activity. The mortality of larvae fed the tassels from NAHA plants from the first and second series of Hi-II assays was significantly negatively correlated with the NAHA histochemical ratings of

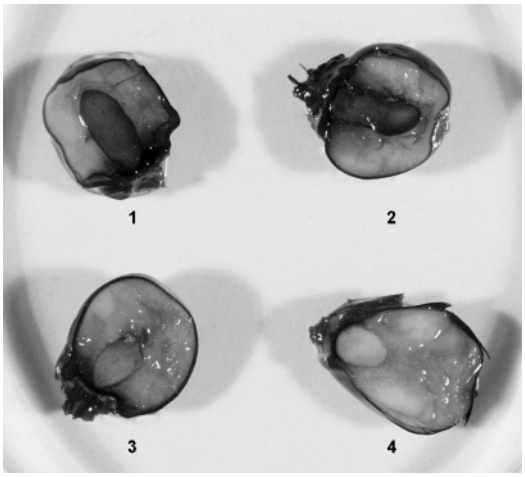

Figure 2. Histochemical detection of NAHA activity in kernels of Oh43 transformants. Key: 1–3, kernels from transformants that had high activity against *S. frugiperda* with strong staining for NAHA in the germ 4; untransformed Oh43.

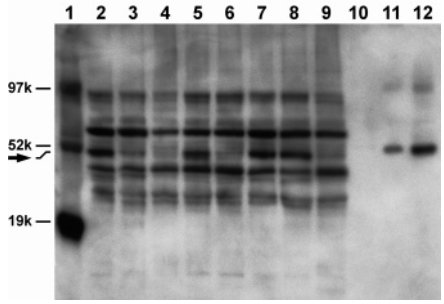

Figure 3. Antibody detection of NAHA in leaf disks of Oh43 transformants. Lane 1, molecular weight markers; lanes 2–9, leaf extracts of transformants; and lanes 10–12, jack bean NAHA at 12, 24, and 60 ng of protein, respectively. The arrow indicates *Arabidopsis* NAHA.

tassel tissue ($R = -0.61$, $P = 0.0006$, $F = 15.16$, df 1,26; $R = -0.35$, $P = 0.0022$, $F = 10.08$, df 1,74; respectively). The larval feeding damage on Oh43 leaf disks was significantly negatively correlated ($R = -0.34$, $P = 0.02$, $F = 5.70$, df 1,47) with levels of NAHA determined with antibody assays once two obvious graphical outliers were removed. Batch to batch variability appeared to influence the degree of correlation, as the *R* for a single batch was −0.72 for feeding vs NAHA levels and −0.52 for mean weights of survivors vs NAHA levels. Further statistical analyses did not demonstrate any apparent indication of threshold or plateau dose effects over the range of NAHA concentrations obtained in the plants.

Detection of NAHA DNA and RNA in Transformants. Full length NAHA genes were detected in most of the Hi-II and Oh43 transformants (**Figure 4**). The sequence of representative PCR products matched the sequence of the introduced gene for both series of transformants. The cDNA obtained from RNA from Oh43 leaf disks of representative transformant lines with activity against insects examined also yielded a band of the correct length and only a Maz band corresponding to the intron-excised cDNA (**Figure 5**), indicating that the PCR products were based only on cDNA generated from mRNA. Southern blot analyses generally indicated a low copy number in representative more active Hi-II and Oh43 transformants and a higher copy number in representative positive Hi-II and Oh43 gene positive transformants that did not have much activity against insects (**Figure 6**).

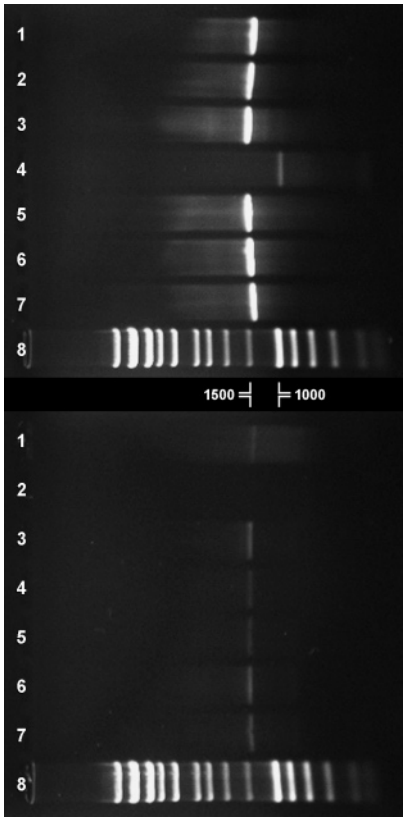

Figure 4. Detection of the introduced *Arabidopsis* NAHA gene by PCR in Hi-II (upper gel) and Oh43 (lower gel). Lanes 1–7 are PCR products from genomic DNA, and lane 8 is molecular weight markers. A truncated product is seen in lane 4 of the upper gel, and a null detection is seen in lane 2 of the lower gel.

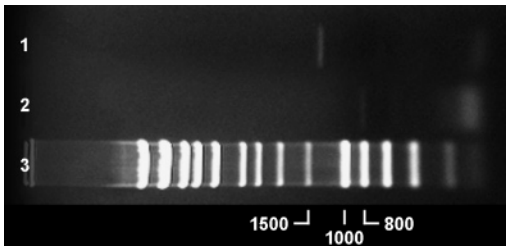

Figure 5. Detection of the mRNA of the introduced *Arabidopsis* NAHA gene in Oh43. Lane 1, primers for NAHA used; lane 2, primers for Maz used; and lane 3, molecular weight markers.

DISCUSSION

NAHA Properties. A diversity of NAHAs have been described from plants. The pI values reported have ranged from near neutral for fenugreek (*Trigonella foenum graecum*) (6.78, 6.3) (*31*) and lupine (*Lupinus luteus*) (7.05) (*32*) to acidic for wheat (4.5) (*33*), fenugreek (4.9, 4.65) (*31*), and jack bean (4.8) (*34*). The *Arabidopsis* NAHA in the present study had a predicted pI of 5.55 (based on DNA-Star software predictions) and an actual pI of ca. 4.8 when expressed in maize. The NAHA of cabbage, which is in the same family as *Arabidopsis*, had a pI of 6.0 (*35*). The difference in pI is not unexpected as multiple genes putatively coding for *Arabidopsis* NAHA are in Genbank, and multiple NAHAs with varying pI values have been reported from the same species as just described. A thermostable form of NAHA isolated from *A. niger* had a pI of 4.4 and could act on *p*-nitrophenyl-NAGA (*36*). Interestingly, the more acidic forms from fenugreek also have a greater preference for naphthol and methylumbelliferyl NAGA than *N,N*-diacetyl-chitobiose

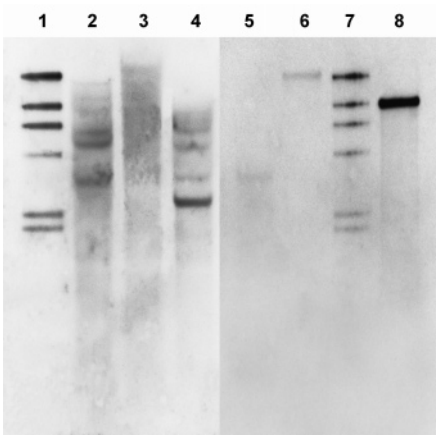

Figure 6. Southern blots indicating introduced *Arabidopsis* NAHA gene copies in Oh43 (B). Lanes 1 and 7, 2.5 and 5 ng, respectively, of molecular weight markers 23130, 9420, 6560, 4360, 2320, and 2030; lanes 2–4, 15 μg of genomic DNA cut with SstI, SmaI, and EcoRI, respectively, from transformant line with lower activity against *S. frugiperda* showing multiple copies of the NAHA gene; lane 5, 20 μg of genomic DNA cut with EcoRI from transformant line with higher activity against *S. frugiperda* showing one copy of the NAHA gene; lane 6, 15 μg of *Arabidopsis* genomic DNA cut with SstI; and lane 8, 1 ng of NAHA construct used as a positive control.

derivatives (*31*). The molecular weights for the fenugreek monoforms range from 24000 to 30000 (*31*) and 29000 for apple *Malus domestica* (*37*), which is smaller than the predicted size of ca. 51000 for the *Arabidopsis* gene used in the present study (which does not include potential additions in size due to potential glycosylation). Expressed NAHA from cabbage had three subunits of 51000, 57000, and 64000 molecular weights (*35*).

Activity of Chitin Degrading Enzymes. A fungus-derived NAHA gene has been cloned and introduced into plants, thereby increasing fungal resistance (*13*). Insect chitinases that have been cloned (*38*) have been expressed in tobacco and increased insect resistance (*39*), despite some processing errors (*40*). A streptomycete chitinase and chitobiosidase expressed in plants also increased resistance to insects (*41*). However, these microbial chitinases can cause plant growth abnormalities (*18*, *19*). Although plant chitinases have been cloned and expressed to increase insect resistance, only recently has a plant (poplar)-derived chitinase introduced into tomato resulted in enhanced insect resistance, with no reported plant growth abnormalities (*17*). We believe our report to be the first utilizing a NAHA from any source to increase insect resistance. No obvious plant abnormalities were noted in the more active, higher expressing plants of either Hi-II or Oh43. Although chitin is an obvious target, interaction with maize chitinases already present and/or targeting other membranes/proteins that are subject to *N*-acetyl deglycoslyation may also be involved in increasing resistance to the insects examined. Resistance appears to potentially involve more than one group of insects (i.e., both caterpillars and beetles), but further study is needed to better determine the potential range of maize feeding or other insects to which the NAHA gene may confer resistance. Introduction of plant-derived genes is likely to be more acceptable to consumers than introduction of genes from microbial or animal sources (*42*). It is possible that we may be able to increase activity against insects by combining the NAHA with a plant chitinase, which may also help explain why plant chitinases are generally not active against insects. However, as indicated previously, certain levels of chitinase expression can reduce the vigor of some

plants (*13*). Combinations of genes that target different functional classes may also be feasible (*15*). Our continuing studies will explore these possibilities. Increasing insect resistance cannot only result in higher yields but also better quality products due to indirect reduction of mycotoxin levels (*2*, *3*).

ABBREVIATIONS USED

BMS, black Mexican sweet; Bt, *Bacillus thuringiensis*; β-GUS, glucuronidase; NAGA, *N*-acetyl β-D-glucosamide; NAHA, β-*N*-acetyl hexosaminidase; PAG, polyacryamide gel; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate.

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LITERATURE CITED

(1) Oerke, E. C. *Crop Production and Crop Protection: Estimated Losses in Major Food and Cash Crops*; Elsevier: Amsterdam, 1994.

(2) Dowd, P. F. The involvement of arthropods in the establishment of mycotoxigenic fungi under field conditions. In *Mycotoxins in Agriculture and Food Safety*; Sinha, K., Bhatnagar, D., Eds.; Marcel Dekker: New York, 1998; pp 307−350.

(3) Dowd, P. F. Insect management to facilitate preharvest mycotoxin management. *Toxicol. Rev.* **2003**, *22*, 327−350.

(4) Bakan, B.; Melcionn, D.; Richard-Molard, D.; Cahagneir, B. Fungal growth and *Fusarium* mycotoxin content in isogenic traditional maize and genetically modified maize grown in France and Spain. *J. Agric. Food Chem.* **2002**, *50*, 728−731.

(5) Clements, M. J.; Campbell, K. W.; Maragos, C. M.; Pilcher, C.; Headrick, J. M.; Pataky, J. K.; White, D. G. Influence of CryIAb protein and hybrid genotype on fumonisin contamination and *Fusarium* ear rot of corn. *Crop Sci.* **2003**, *43*, 1283−1293.

(6) Dowd, P. F. Indirect reduction of ear molds and associated mycotoxins in Bt corn under controlled and open field conditions in Illinois: Potential utility and limitations. *J. Econ. Entomol.* **2000**, *86*, 1669−1679.

(7) Dowd, P. F. Biotic and abiotic factors limiting efficacy of Bt corn in indirectly reducing mycotoxin levels in commercial fields. *J. Econ. Entomol.* **2001**, *94*, 1067−1074.

(8) Munkvold, G. P.; Hellmich, R. L.; Rice, L. G. Comparison of fumonisin concentrations in kernels of transgenic Bt maize hybrids and non-transgenic hybrids. *Plant Dis.* **1999**, *83*, 130−138.

(9) Gould, F. Sustainability of transgenic insecticidal cultivars: Integrating pest genetics and ecology. *Annu. Rev. Entomol.* **1998**, *43*, 701−726.

(10) Shelton, A. M.; Zhao, J.-Z.; Roush, R. T. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. *Annu. Rev. Entomol.* **2002**, *47*, 845−879.

(11) Peairs, F. B. *Managing Corn Pests with Bt Corn*; Colorado State University Cooperative Extension Bulletin #0.708; Colorado State University: Ft. Collins, CO, 2006; www.ext.colostate.edu/Pubs/crops/00708.html.

(12) Ferré, J.; Van Rie, J. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annu. Rev. Entomol.* **2002**, *47*, 501−535.

(13) Bolar, J. P.; Norelli, J. L.; Harman, G. E.; Brown, S. K.; Aldwinckle, H. S. Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants. *Transgenic Res.* **2001**, *10*, 533−543.

(14) Dowd, P. F.; Johnson, E. T.; Williams, W. P. Strategies for insect management targeted toward mycotoxin management. In *Aflatoxin and Food Safety*; Abbas, H. K., Ed.; CRC Press: Boca Raton, FL, 2005; pp 517−542.

(15) Dowd, P. F. A functional approach to more effective multigenic host plant pest resistance. *Mycopathologia* **2002**, *155*, 46.

(16) Kramer, K. L.; Muthukrishnan, S.; Johnson, L.; White, F. Chitinases for insect control. In *Advances in Insect Control, The Role of Transgenic Plants*; Carozzi, N., Koziel, M., Eds.; Taylor and Francis: Bristol, PA, 1997; pp 185−193.

(17) Lawrence, S. D.; Novak, N. G. Expression of poplar chitinase in tomato leads to inhibition of development in Colorado potato beetle. *Biotechnol. Lett.* **2006**, *28*, 593−599.

(18) Bolar, J. P.; Norelli, J. L.; Wong, K.-W.; Hayes, C. K.; Harman, G. E.; Aldwinckle, H. S. Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigor. *Phytopathology* **2000**, *90*, 72−77.

(19) Gongora, C. E.; Broadway, R. M. Plant growth and development influenced by transgenic insertion of bacterial chitinolytic enzymes. *Mol. Breed.* **2002**, *9*, 123−135.

(20) Moss, G. P. International Union of Biochemistry and Molecular Biology. *Enzyme Nomenclature*; Queen May University: London, 2006; http//:www.chem.qmul.ac.uk/iubmb/ enzyme.

(21) Dowd, P. F.; Bartelt, R. J.; Behle, R. W.; Berhow, M. A.; Duvick, J. P.; Kendra, D. F.; Lagrimini, L. M.; McGuire, M. R.; Molid, G.; White, D. G. Advances in insect-oriented IPM of mycotoxigenic fungi of midwest corn: FY-1999. In *Proceedings of the Aflatoxin Elimination Workshop*; Robens, J. F., Cary, J. W., Eds.; U.S. Department of Agriculture: Beltsville, MD, 1999; pp 29−30.

(22) Dowd, P. F. Toxicological and biochemical interactions of the fungal metabolites fusaric acid and kojic acid with xenobiotics in *Heliothis zea* (F.) and *Spodoptera frugiperda* (J.E. Smith). *Pestic. Biochem. Physiol.* **1988**, *32*, 123−134.

(23) Dowd, P. F.; Zuo, W.-N.; Gillikin, J. W.; Johnson, E. T.; Boston, R. S. Enhanced resistance to *Helicoverpa zea* in tobacco expressing an activated form of maize ribosome-inactivating protein. *J. Agric. Food Chem.* **2003**, *51*, 3568−3574.

(24) Dowd, P. F.; Johnson, E. T. Association of a specific cationic peroxidase isozyme with maize stress and disease resistance responses, genetic identification, and identification of a cDNA coding for the isozyme. *J. Agric. Food Chem.* **2005**, *53*, 4464−4470.

(25) Dowd, P. F.; Holmes, R. A.; Pinkerton, T. S.; Johnson, E. T.; Lagrimini, L. M.; Boston, R. S. Relative activity of a tobacco hybrid expressing high levels of a tobacco anionic peroxidase and maize ribosome-inactivating protein against *Helicoverpa zea* and *Lasioderma serricorne*. *J. Agric. Food Chem.* **2006**, *54*, 2629−2634.

(26) Peterbauer, C. K.; Lorito, M.; Hayes, C. K.; Harman, G. E.; Kubicek, C. P. Molecular cloning and expression of the *nag1* gene (*N*-acetyl-β-D-glucosaminidase-encoding gene) from *Trichoderma harzianum* P1. *Curr. Genet.* **1996**, *30*, 325−331.

(27) Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **1975**, *98*, 503−517.

(28) Muhitch, M. J.; Hua, L.; Rastogi, R.; Sollenberger, K. G. Isolation of a promoter sequence from the glutamine synthase$_{1-2}$ gene capable of conferring tissue specific gene expression in transgenic maize. *Plant Sci.* **2002**, *163*, 865−872.

(29) Dowd, P. F. Enhanced maize (*Zea mays* L.) pericarp browning: Associations with insect resistance and involvement of oxidizing enzymes. *J. Chem. Ecol.* **1994**, *20*, 2777−2803.

(30) SAS Institute. *SAS for Windows*, Version 8.0; SAS Institute: Cary, NC, 1999.

(31) Bouquelet, S.; Spik, G. Properties of four molecular forms of *N*-acetyl-β-D-hexosaminidase isolated from germinating seeds

of fenugreek (*Trigonella foenum graecum*). *Eur. J. Biochem.* **1978**, *84*, 551−559.
(32) Pócsi, I.; Kiss, L.; Nánási, P. Studies on the *N*-acetyl-β-D-hexosaminidase B from germinating *Lupinus luteus* L. seeds. I. Purification and characterization. *Biochim. Biophys. Acta* **1990**, *1039*, 110−118.
(33) Carratu, G.; Colacino, C.; Conti, S.; Giannattasio, M. β-*N*-Acetylglucosaminidase and β-galactosidase from aleurone layers of resting wheat grains. *Phytochemistry* **1985**, *24*, 1465−1469.
(34) Li, S.-C.; Li, Y.-T. Studies on the glycosidases of jack bean meal. III. Crystalization and properties of β-*N*-acetylhexosaminidase. *J. Biol. Chem.* **1970**, *245*, 5153−5160.
(35) Chang, C. T.; Young, F. P.; Chang, M. H.; Sung, H. Y. Purification and properties of β-*N*-acetylhexosaminidase from cabbage. *Biochem. Mol. Biol. Int.* **1998**, *45*, 371−380.
(36) Pera, L. M.; Infante Majolli, M. V.; Baigori, M. D. Purification and characterization of a thermostable and highly specific β-*N*-acetyl-D-glucosaminidase from *Aspergillus niger* 149. *Biotechnol. Appl. Biochem.* **1997**, *26*, 183−187.
(37) Choi, S. Y.; Gross, K. C. 1994. *N*-Acetyl-beta-D-glucosaminidase from "Golden Delicious" apples. *Phytochemistry* **1994**, *36*, 1−6.
(38) Zen, K.-C.; Choi, H. K.; Krishnamachary, N.; Muthukrishnan, S.; Kramer, K. J. Cloning, expression and hormonal regulation of an insect β-*N*-acetylglucosaminidase gene. *Insect Biochem. Mol. Biol.* **1996**, *26*, 435−444.
(39) Ding, X.; Gopalakrisnan, B.; Johnson, L. B.; White, F. F.; Wang, X.; Morgan, T. D.; Kramer, K. J.; Muthukrishnan, S. Insect resistance of transgenic tobacco expressing an insect chitinase gene. *Transgenic Res.* **1998**, *7*, 77−84.
(40) Wang, X.; Ding, X.; Gopalakrisnan, B.; Borgan, T. D.; Johnson, L.; White, F. F.; Muthukrishnan, S.; Kramer, K. J. Characterization of a 46 kDa insect chitinase from transgenic tobacco. *Insect Biochem. Mol. Biol.* **1996**, *26*, 1055−1064.
(41) Gongora, C. E.; Wang, S.; Barbehenn, R. V.; Broadway, R. M. Chitinolytic enzymes from *Streptomyces albidoflavus* expressed in tomato plants: Effects on *Trichoplusia ni*. *Entomol. Exp. Appl.* **2001**, *99*, 193−204.
(42) National Research Council, Committee on Genetically Modified Pest-Protected Plants, Board on Agricultural and Natural Resources. *Genetically Modified Pest-Protected Plants: Science and Regulation*; National Academy Press: Washington, DC, 2000.

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